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US 20020182229A1

(19) United States

(12) Patent Application Publication (10) Pub. No.: US 2002/0182229 A1

Brown et al. (43) Pub. Date: Dec. 5, 2002

(54) ANAEROBE TARGETED ENZYME MEDIATED PRODRUG THERAPY

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(73) Assignee: The Board of Trustees of the Leland Stanford Junior University

(21) Appl. No.: 10/151,069

(22) Filed: May 17, 2002

Related U.S. Application Data

(63) Continuation of application No. 08/686,502, filed on Jul. 24, 1996, now Pat. No. 6,416,754, which is a continuation of application No. 08/465,932, filed on Jun. 6, 1995, now abandoned, which is a continuation of application No. 08/227,313, filed on Apr. 13, 1994, now abandoned, which is a continuation of application No. 08/206,430, filed on Mar. 3, 1994, now abandoned.

Publication Classification

(51) Int. Cl.[7] A61K 39/08; C12N 15/74

(52) U.S. Cl. 424/247.1; 435/320.1

(57) ABSTRACT

A genetically-engineered anaerobic organism is provided which, under anaerobic conditions present in a solid tumor, produces an enzyme capable of catalyzing the conversion of a prodrug to its highly cytotoxic product in situ and methods of treating tumors using same.

PGPUB-DOCUMENT-NUMBER: 20020182229
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020182229 A1

TITLE: Anaerobe targeted enzyme mediated prodrug therapy

PUBLICATION-DATE: December 5, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Brown, John Martin	Redwood City	CA	US
Minton, Nigel P.	Salisbury	CA	GB
Giaccia, Amato	Stanford		US

US-CL-CURRENT: 424/247.1; 435/320.1

CLAIMS:

We claim:

1. A vector expressed in obligate anaerobes for the production of an enzyme capable of converting a non toxic prodrug to a toxic chemotherapeutic agent.

2. The vector of claim 1, wherein the anaerobe is a member of the genus Clostridium.

3. The vector of claim 1, wherein the enzyme is nitroreductase.

4. The vector of claim 3, wherein the enzyme is nitroreductase and the anaerobe is a member of the genus Clostridium.

5. The vector of claim 4, wherein the anaerobe is Clostridium acetobutylicum.

6. The vector of claim 5, wherein the vector is an anaerobe vector comprising the ntr gene encoding E. coli B nitroreductase (NTR) and the promoter and RBS of the ferredoxin (Fd) gene of Clostridium pasteurianum.

7. The vector of claim 1, wherein the enzyme is .beta.-glucuronidase.

8. The vector of claim 7, wherein the anaerobe is a member of the genus Clostridium.

9. The vector of claim 8, wherein the anaerobe is Clostridium acetobutylicum.

10. The vector of claim 1, wherein the enzyme is cytosine deaminase.

11. The vector of claim 10, wherein the anaerobe is a member of the genus Clostridium.

12. The vector of claim 11, wherein the anaerobe is Clostridium acetobutylicum.

13. The vector of claim 2, wherein the prodrug is CB1954.

14. The vector of claim 3, wherein the prodrug is selected from the group consisting of glucuronides of epirubicin, 5-fluorouracil, and 4-hydroxycyclophosphamide.

15. The vector of claim 4, wherein the prodrug is 5-fluorocytosine.

16. A method of targeting a toxic chemotherapeutic agent to a tumor in a tumor-bearing individual comprising the steps of: a) administering an effective amount of a genetically engineered anaerobic microorganism capable of proliferating and producing an enzyme in the hypoxic/necrotic environment of a tumor to said individual; and then b) systemically administering a prodrug which is converted at the site of the tumor to the toxic chemotherapeutic agent by the enzyme produced by the microorganism.

17. The method of claim 16, wherein the anaerobic microorganism is a member of the genus Clostridium.

18. The method of claim 17, wherein the anaerobic microorganism is Clostridium acetobutylicum.

19. The method of claim 16, wherein the enzyme is nitroreductase.

20. The method of claim 16, wherein the prodrug is CB1954.

21. The method of claim 16, wherein the anaerobic microorganism is Clostridium acetobutylicum, the enzyme is E. coil B nitroreductase (NTR) and the prodrug is CB1954.

22. The method of claim 16, wherein the enzyme is .beta.-glucuronidase.

23. The method of claim 16, wherein the prodrug is selected from the group consisting of glucuronides of epirubicin, 5-fluorouracil, and 4-hydroxycyclophosphamide.

24. The method of claim 16, wherein the anaerobic microorganism is Clostridium acetobutylicum, the enzyme is .beta.-glucuronidase, and the prodrug is selected from the group consisting of glucuronides of epirubicin, 5-fluorouracil, and 4-hydroxycyclophosphamide.

25. The method of claim 16, wherein the enzyme is cytosine deaminase.

26. The method of claim 16, wherein the prodrug is 5-fluorocytosine.

27. The method of claim 16, wherein the anaerobic microorganism is Clostridium acetobutylicum, the enzyme is cytosine deaminase, and the prodrug is 5-fluorocytosine.

US006939548B2

(12) **United States Patent**
Wilkins et al.

(10) **Patent No.: US 6,939,548 B2**
(45) **Date of Patent: Sep. 6, 2005**

(54) **METHODS TO PRODUCE HIGH LEVELS OF *C. DIFFICILE* TOXINS**

(75) Inventors: **Tracy D. Wilkins**, Riner, VA (US); **David M. Lyerly**, Radford, VA (US); **J. Scott Moncrief**, Christiansburg, VA (US); **Limin Zheng**, Blacksburg, VA (US); **Carol Phelps**, Floyd, VA (US)

(73) Assignee: **Techlab, Inc.**, Blacksburg, VA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 372 days.

(21) Appl. No.: **10/222,038**

(22) Filed: **Aug. 15, 2002**

(65) **Prior Publication Data**

US 2003/0129198 A1 Jul. 10, 2003

Related U.S. Application Data

(62) Division of application No. 09/545,773, filed on Apr. 10, 2000, now Pat. No. 6,733,760.
(60) Provisional application No. 60/190,111, filed on Mar. 20, 2000, provisional application No. 60/186,201, filed on Mar. 1, 2000, and provisional application No. 60/128,686, filed on Apr. 9, 1999.

(51) **Int. Cl.**[7] **A61K 39/08**; A61K 49/00; A61K 39/00; A61K 39/02

(52) **U.S. Cl.** **424/247.1**; 424/9.1; 424/9.2; 424/184.1; 424/185.1; 424/192.1; 424/200.1; 424/234.1; 530/300; 530/350; 536/23.7

(58) **Field of Search** 424/9.1, 9.2, 184.1, 424/185.1, 192.1, 200.1, 234.1, 247.1; 530/300, 350; 536/23.7

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Primary Examiner—Rodney P. Swartz
(74) *Attorney, Agent, or Firm*—Morrison & Foerster LLP

(57) **ABSTRACT**

The present invention relates to the field of medical immunology and further to pharmaceutical compositions, methods of making and methods of use of vaccines. More specifically this invention relates to recombinant proteins derived from the genes encoding *Clostridium difficile* toxin A and toxin B, and their use in an active vaccine against *C. difficile*.

16 Claims, 11 Drawing Sheets

DOCUMENT-IDENTIFIER: US 6939548 B2
TITLE: Methods to produce high levels of C. difficile toxins

CLAIMS:

1. A method to produce the repeating unit portion of Clostridium difficile toxin A (rARU) or toxin B (rBRU) in high yield in E. coli bacteria which comprises culturing said bacteria under selective pressure, wherein said bacteria have been modified to contain a nucleic acid comprising an expression system which comprises a nucleotide sequence encoding said rARU or rBRU operably linked to an inducible promoter, whereby said rARU or rBRU is produced at levels of at least 10 mg/l of culture.

11. A method to produce the repeating unit portion of Clostridium difficile toxin A (rARU) in high yield in E. coli bacteria which comprises culturing said bacteria under selective pressure, wherein said bacteria have been modified to contain a nucleic acid comprising an expression system which comprises a nucleotide sequence encoding said rARU operably linked to an inducible promoter, whereby said rARU is produced at levels of at least 10 mg/l of culture.

US005955368A

United States Patent [19]

Johnson et al.

[11] Patent Number: 5,955,368

[45] Date of Patent: Sep. 21, 1999

[54] **EXPRESSION SYSTEM FOR CLOSTRIDIUM SPECIES**

[75] Inventors: **Eric A. Johnson**; **Marite Bradshaw**, both of Madison, Wis.; **Julian I. Rood**, Bentleigh; **Dena Lyras**, Heidelberg Heights, both of Australia

[73] Assignee: **Wisconsin Alumni Research Foundation**, Madison, Wis.

[21] Appl. No.: **09/056,075**

[22] Filed: **Apr. 6, 1998**

[51] **Int. Cl.**[6] C12N 1/21; C12N 15/70; C12N 15/74; C12N 15/64

[52] **U.S. Cl.** **435/488**; 435/320.1; 435/476; 435/252.3; 536/23.1; 536/24.1

[58] **Field of Search** 435/320.1, 172.3, 435/488, 476, 252.3; 536/23.1, 24.1

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Primary Examiner—Johnny F. Railey, II

Attorney, Agent, or Firm—Quarles & Brady LLP

[57] **ABSTRACT**

A system is used to express clostridial gene constructions in a clostridial host. A mobilizable transfer plasmid is described which permits the direct transfer of the plasmid, and genes carried on it, from *E. coli* into Clostridium species. A promoter is described for use in clostridial species. Also, a useful host strain is used which is nontoxigenic and which permits high levels of expression of clostridial genes using the clostridial promoter.

15 Claims, 2 Drawing Sheets

DOCUMENT-IDENTIFIER: US 5955368 A
TITLE: Expression system for clostridium species

CLAIMS:

6. A method for the delivery and expression of genetic constructs in a Clostridium species comprising the steps of:

making a genetic construction including a promoter effective in the Clostridium species;

inserting the genetic construction in a mobilizable transfer plasmid which includes an origin of replication effective in E. coli; an origin of replication effective in a Clostridium species; a gene for an antibiotic resistance marker; and an origin of conjugative transfer which, when actuated, is capable of directing the transfer of the plasmid from E. coli into a Clostridium species;

transforming the mobilizable plasmid into an E. coli strain;

culturing the E. coli strain carrying the plasmid with a culture of the Clostridium species under conditions which facilitate conjugative transfer of the plasmid; and

selecting for bacteria of the Clostridium species which are hosting the plasmid.

8. A method as claimed in claim 6 wherein the promoter effective in Clostridium species is the NTNH promoter from Clostridium botulinum.

DOCUMENT-IDENTIFIER: US 5496725 A
TITLE: Secretion of Clostridium cellulase by E. coli

CLAIMS:

1. A recombinant microorganism comprising a vector for the heterologous expression of a cellulase enzyme wherein said enzyme is capable of digesting chemically or enzymatically untreated natural plant materials and said enzyme has a molecular weight of about 58,000 daltons as measured by gel exclusion chromatography wherein said microorganism comprises:

(a) an expression vector comprising a DNA sequence coding for said cellulase enzyme; and

(b) at least one DNA sequence coding for a signal sequence useful for the secretion of said cellulase enzyme, wherein a first DNA sequence of the expression vector codes for cellulase obtained from Clostridium strain IY-2.

3. A microorganism of claim 2 wherein said 2.8 kb insert is further cleaved into 2.2 kb DNA fragment comprising at least an entire structural gene and signal sequences of cellulase and the promoter is from the Clostridium cellulase gene on plasmid pPC1A.

US005496725A

United States Patent [19]

Yu

[11] Patent Number: 5,496,725

[45] Date of Patent: Mar. 5, 1996

[54] **SECRETION OF CLOSTRIDIUM CELLULASE BY *E. COLI***

[76] Inventor: **Ida K. Yu**, 3202 Monte Vista Pl., Davis, Calif. 95616

[21] Appl. No.: **105,870**

[22] Filed: **Aug. 11, 1993**

[51] **Int. Cl.**[6] **C12N 1/15**; C12N 1/21; C12N 5/10; C12N 9/42

[52] **U.S. Cl.** **435/252.3**; 435/209; 435/240.2; 435/252.33; 435/254.11; 435/320.1

[58] **Field of Search** 435/252.2, 252.8, 435/69.1, 842

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Attorney, Agent, or Firm—Albert P. Halluin; Pennie & Edmonds

[57] **ABSTRACT**

A gene, encoding an endocellulase from a newly isolated mesophilic Clostridium strain IY-2 which can digest bamboo fibers, cellulose, rice straw, and sawdust, was isolated by shotgun cloning in an *E. coli* expression plasmid pLC2833. *E. coli* positive clones were selected based on their ability to hydrolyze milled bamboo fibers and cellulose present in agar plates. One clone contained a 2.8 kb DNA fragment that was responsible for cellulase activity. Western blot analyses indicated that the positive clone produced a secreted cellulase with a mass of about 58,000 daltons that was identical in size to the subunit of one of the three major Clostridium cellulases. The products of cellulose digestion by this cloned cellulase were cellotetraose and soluble higher polymers. The cloned DNA contained signal sequences capable of directing the secretion of heterologous proteins from an *E. coli* host. The invention describes a bioprocess for the treatment of cellulosic plant materials to produce cellular growth substrates and fermentation end products suitable for production of liquid fuels, solvents, and acids.

5 Claims, 7 Drawing Sheets

DOCUMENT-IDENTIFIER: US 5177017 A
TITLE: Molecular cloning of the genes responsible for collagenase production from Clostridium histolyticum

CLAIMS:

4. The DNA fragment of claim 2 wherein the DNA derived from C. histolyticum contains a promoter within the inserted Clostridium DNA such that the DNA can be transcribed under control of the promoter to yield mRNA capable of being translated to yield the polypeptide of about 68,000 daltons without the functioning of a promoter external to the DNA derived from C. histolyticum.

6. A DNA fragment comprising the DNA fragment of claim 4 fused contiguously at least a portion of the structural gene for E. coli .beta.-galactosidase, the portion of the structural gene for .beta.-galactosidase being operatively linked to a lac promoter such that:

(i) the DNA derived from Clostridium can be transcribed under control of the promoter located within the Clostridium DNA to yield mRNA capable of being translated to yield the polypeptide of about 68,000 daltons without the functioning of a promoter external to the DNA derived from C. histolyticum; and

(ii) the Clostridium DNA fused to the DNA sequence comprising at least a portion of the structural gene for E. coli .beta.-galactosidase can be transcribed under control of the lac promoter to yield mRNA capable of being translated to yield a fusion polypeptide of molecular weight greater than 70,000 daltons displaying the antigenicity of C. histolyticum collagenase and containing at least a portion of the amino acid sequence of E. coli .beta.-galactosidase.

13. An isolated and purified recombinant DNA fragment comprising:

(a) a Clostridium histolyticum structural gene encoding C. histolyticum collagenase and capable of being transcribed to yield mRNA capable of being translated to yield polypeptides of molecular weight of from about 70,000 dalonts to about 100,000 daltons and having the enzymatic activity of C. histolyticum collagenase; and

(b) an internal promoter located within the structural gene of (a) and operatively linked to a Clostridium DNA sequence comprising a portion of the structural gene of (a) and capable of being transcribed to yield mRNA capable of being translated to yield a polypeptide of about 68,000 daltons molecular weight, the polypeptide having collagenase activity, the polypeptide being distinguishable by the heterologous expression of the sequence of claim 3 from the endogenous production by C. histolyticum of multiple forms of collagenase by the essential absence of those forms of C. histolyticum collagenease having molecular weights of above about 70,000 daltons determined by the expression of the C. histolyticum genomic coding sequence; the internal promoter being positioned such that the DNA sequence of (b) can be translated independently of the structural gene of (a).

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United States Patent [19]

Lin et al.

[11] Patent Number: 5,177,017

[45] Date of Patent: Jan. 5, 1993

[54] **MOLECULAR CLONING OF THE GENES RESPONSIBLE FOR COLLAGENASE PRODUCTION FROM *CLOSTRIDIUM HISTOLYTICUM***

[75] Inventors: **Hun-Chi Lin; Shau-Ping Lei**, both of Los Angeles, Calif.

[73] Assignee: **Trigen, Inc.**, Santa Monica, Calif.

[21] Appl. No.: **498,919**

[22] Filed: **Mar. 22, 1990**

[51] **Int. Cl.**[5] **C12N 15/57**; C12N 15/70; C12N 15/31

[52] **U.S. Cl.** **435/252.33**; 435/220; 435/320.1; 935/14; 935/29; 935/73; 536/23.7

[58] **Field of Search** 435/69.1-69.9, 435/320.1, 220, 252.33; 536/27

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(List continued on next page.)

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Attorney, Agent, or Firm—Poms, Smith, Lande & Rose

[57] **ABSTRACT**

Genetically engineered *E. coli* carry vectors containing inserts that code for *Clostridium histolyticum* collagenase. These inserts code for: (a) a form of collagenase having a molecular weight of about 68,000 daltons in the essential absence of larger forms of collagenase; (b) the 68 kd form of collagenase and a fusion polypeptide consisting of the collagenase protein fused to at least a portion of the β-galactosidase protein of *E. coli;* or (3) the 68 kd form of collagenase and polypeptides of molecular weight of from above about 68,000 daltons to about 100,000 daltons and having the enzymatic activity of *C. histolyticum* collagenase as indicated by digestion of ^{3}H-acetylated collagen and by specific inhibition by 1,10-phenanthroline plus EDTA. The collagenase genes in the transformed *E. coli* are expressed efficiently in the transformed cells to yield enzymatically active and immunologically cross-reactive collagenase. In particular, the 68 kd form of collagenase is resistant to autocatalytic degradation and is stable to long-term storage. Genetically engineered collagenase, especially the 68 kd form that is resistant to autocatalytic degradation, can be used for isolation of pancreatic islets, for the isolation of dispersed tumor cells, or for treatment of "slipped disc."

23 Claims, 8 Drawing Sheets